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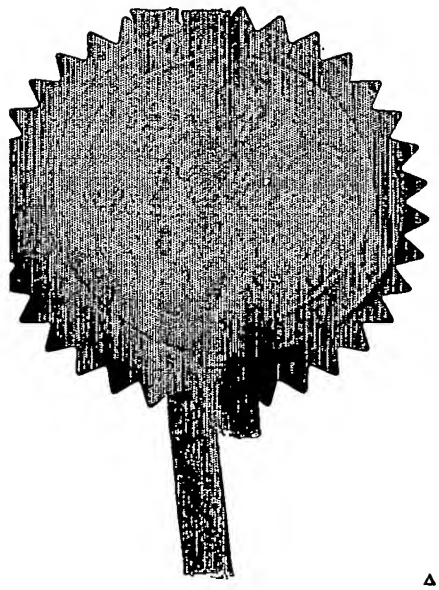

Signed R. Mahoney

Dated 10 JUL 2003

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Your reference	AVH/P05965UK
Patent application number *(The Patent Office will fill in this part)*	0212622.5
	31 MAY 2002
	31MAY02 E722918-2 D00351 SYSTEM P01/7700 0.00-0212622.5
Full name, address and postcode of the or of each applicant *(underline all surnames)*	Natural Technologies ~~Group~~ Limited Holly Farm Business Park Honiley, Near Kenilworth, Warwickshire, CV8 1NP.
Patents ADP number *(if you know it)*	8394785001
If the applicant is a corporate body, give the country/state of its incorporation	GB
Title of the invention	Bacterial Transforming Agent
Name of your agent *(if you have one)*	**Lewis & Taylor**
"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	**5 The Quadrant** **Coventry** **CV1 2EL**
Patents ADP number *(if you know it)*	**711001**

If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

If this application is divided or otherwise derived from an earlier UK application, give the number and filing date of the earlier application	Number of earlier application	Date of filing *(day / month / year)*

Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'yes' if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is not named as an applicant, or* *c) any named applicant is a corporate body.* *See note (d))*	Yes

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Continuation sheets of this form	-
Description	28
Claim(s)	-
Abstract	-
Drawing(s)	-

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Priority documents	-
Translation of priority documents	-
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	-
Request for preliminary examination and search *(Patents Form 9/77)*	-
Request for substantive examination *(Patents Form 10/77)*	-
Any other documents *(please specify)*	-

11. I/We request the grant of a patent on the basis of this application.

Signature

Date
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A V Hallam
(024) 7622 2756/7145

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BACTERIAL TRANSFORMING AGENT

The present invention relates to agents for increasing the sensitivity of bacteria to anti-microbial agents and particularly, but not exclusively, to agents for transforming bacteria resistant to an antimicrobial agent into bacteria having increased sensitivity to that antimicrobial agent.

The global rise of bacteria and other microorganisms resistant to antibiotics and antimicrobials in general, poses a major threat to mankind. Deployment of massive quantities of antimicrobial agents into the human ecosphere during the past 60 years has introduced a powerful selective pressure for the emergence and spread of antimicrobial-resistant bacterial pathogens. Resistant organisms of special epidemiological importance, due to the preponderance of these pathogens to cause cross-infection in hospitals and other health care settings, include methicillin-resistant *Staphylococcus aureus* (MRSA) and other Gram-positive bacteria such as vancomycin-resistant enterococci (VRE) and *Clostridium difficile*, and *Streptococcus pneumoniae* which is becoming increasingly resistant to β-lactams and other antimicrobials, plus Gram-negative rods that produce extended spectrum β-lactamases. As there is resistance to every clinically available antibiotic, particularly amongst recent strains of epidemic MRSA (EMRSA), there is the prospect of a post-antibiotic era where current antimicrobial agents are ineffective.

Staphylococcus aureus

S. aureus is an important cause of community- and hospital-acquired infection and is the second most important cause of septicaemia after *Escherichia coli* and the second commonest cause of line-associated infection and continuous ambulatory peritoneal dialysis peritonitis. *S. aureus* is also a major cause of

bone, joint and skin infection. Overall, *S. aureus* is the commonest bacterial pathogen in modern hospitals and communities. It is also one of the most antimicrobial resistant and readily transmissible pathogens which, on average, may be carried by about a third of the normal human population, thus facilitating world-wide spread of epidemic strains.

Colonisation is a prerequisite for carriage and infection and staphylococci are well known colonisers of skin, wounds and implantable devices. Carriage usually occurs on specific skin sites histologically associated with apocrine glands, mainly the anterior nares (picking area of the nose) and secondarily the axillae and perineum. It has been postulated that *S. aureus* is disseminated from the nose to the hands and thence to other body sites where infection can occur when breaks in the dermal surfaces, by vascular catheterisation or surgical incision, have occurred. Intranasal mupirocin is the mainstay for the eradication of nasal carriage of Methicillin-resistant *S. aureus* (MRSA), which are by nature multiply antibiotic resistant, during hospital outbreaks. In view of the increasing concern about *S. aureus* infection it is imperative that new and reliable treatments for the elimination of carriage of *S. aureus*, are sought.

By the early 1950s, resistance to penicillin, conferred by a penicillinase (= β-lactamase) born on transmissible plasmids, was common in strains of *S. aureus* acquired in hospitals. Alternative antimicrobial agents, namely tetracycline, streptomycin and the macrolides, were introduced, but resistance developed rapidly. The understanding of the chemistry of the β-lactam ring enabled the development of methicillin, a semisynthetic penicillinase-stable isoxazolyl penicillin. Methicillin and the subsequent development of other isoxazolyl semisynthetic agents such as flucloxacillin, cloxacillin and oxacillin, revolutionised the treatment of *S. aureus* infections.

MRSA were first detected in England in 1960 and have since become a well recognised cause of hospital-acquired infection world-wide. MRSA are resistant to all clinically available β-lactams and cephalosporins and readily acquire resistant determinants to other antimicrobial agents used in hospital medicine. Selective pressure has ensured the rise and world-wide spread of MRSA. Outbreaks caused by 'modern' epidemic MRSA (EMRSA) in the UK began during the early 1980s with a strain subsequently characterised as EMRSA-1. There are now 17 epidemic types recognised in the UK and these have steadily risen in prevalence in England and Wales from 1-2% of reported blood and CSF isolates in 1989-92 to 31.7% in 1997. This rise reflects the increasing domination by epidemic strain types 15 and 16. EMRSA are very transmissible and variably acquire resistance to all antimicrobials in addition to those related to methicillin and the β-lactam ring.

Vancomycin-resistance

S. aureus / MRSA

A further sinister development is the ability of some strains to acquire reduced or intermediate resistance to glycopeptides. Glycopeptide antibiotics, vancomycin in particular, have been the drugs of choice, and in many cases the only active agents, for treating infection with MRSA and other resistant Gram-positive bacteria such as enterococci. If MRSA are not controlled, then the clinical use of vancomycin or teicoplanin rises because of the increased number of wound and blood stream infections in hospitalised patients. Soon after Hiramatsu reported vancomycin-intermediate-resistant MRSA in Japan (Lancet 1997, **350**, pp1670-3), than EMRSA-16 began to reduce its sensitivity to vancomycin in some clinical isolates from diabetic foot ulcers. A new

epidemic strain, EMRSA-17 developed from this strain and this has an increased ability to resist vancomycin.

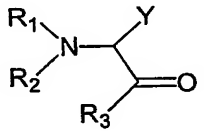
Enterococci

Enterococci, particularly *Enterococcus faecium* and *E. faecalis*, are primarily gut commensals but which can become opportunistic pathogens that colonise and infect immunocompromised hosts, such as liver transplant patients. Vancomycin-resistant *E. faecium* (VREF) emerged and have since become important nosocomial pathogens. Since vancomycin-resistant enterococci first emerged in South London and Paris in 1987, multiply antimicrobial resistant enterococci have been reported with increasing frequency in many countries. Indeed, *E. faecium* resistant to gentamicin, vancomycin and other agents, have caused infections for which no therapeutic agents had been available in the UK, although quinupristin/ dalfopristin, which is active (MIC $\leq$ 2 mg/L) against 86% of *E. faecium* isolates , has now been licensed. In the USA, the proportion of VREF among enterococci isolated from blood cultures increased from 0% in 1989 to 25.9% in 1999. Raw poultry meat appears to be a major source of VREF.

Whilst antimicrobial resistance is of global concern, the only method proposed to control and reduce resistance is by encouraging appropriate use of antimicrobial agents. However, expectations that prudent antibiotic use will deliver reversals in resistance trends should only be accepted with caution. The concept of transforming resistant strains into sensitive ones, with the object of restoring the use of previously established antimicrobial agents rather than develop new agents to which resistance will subsequently develop, has not been explored.

An object of the present invention is to provide a transforming agent for reversing (partially or wholly) the resistance of a bacterial cell to an antimicrobial agent.

The present invention resides in a method of increasing the sensitivity of a bacterial strain to an anti-microbial agent, said method comprising the step of exposing said bacterial strain to a transforming agent having the following formula (I):-



Formula I

where

moieties R_1 and R_2 are each independently selected from, alkyl, alkyloxy, alkyloxycarbonyl, alkylcarbonyloxy, alkenyl, alkenyloxy, alkenyloxycarbonyl, alkenylcarbonyloxy, alkynyl, alkynyloxy, alkynyloxycarbonyl, alkynylcarbonyloxy, each of which may be substituted or unsubstituted, straight chain or branched or cyclic,

aryl, aryloxy, aryloxycarbonyl, arylcarbonyloxy, each of which may be substituted or unsubstituted, and

cabamoyl,

moiety R_3 is selected from alkyl, alkyloxy, alkylcarbonyloxy, alkenyl, alkenyloxy, alkenylcarbonyloxy, alkynyl, alkynyloxy, alkynylcarbonyloxy, each of which may be substituted or unsubstituted, straight chain or branched or cyclic,

aryl, aryloxy, arylcarbonyloxy, each of which may be substituted or unsubstituted, and

carboxyl.

other than R_1, R_2, and R_3 are not all H,
and Y is selected from a natural amino acid side chain.

Sulphur analogues of said oxygen containing substituents are also within the scope of the invention. Reference to cyclic compounds is intended to include heterocyclic compounds having one or more N, S or O atoms in their ring system.

Suitable substituents on any of said R_1, R_2 and R_3 moieties include halogen (eg. F and Cl), hydroxyl (-OH), carboxyl (-CO_2H), amine and amide.

Preferably Y is $-H_2$ (i.e. glycine "side chain")

Preferably, one of R_1 and R_2 is H.

Preferably, one of R_1 and R_2 is alkylcarbonyl (more preferably C_1-C_6 alkylcarbonyl), alkenylcarbonyl (more preferably C_2-C_6 alkenylcarbonyl), alkynylcarbonyl (more preferably C_2-C_6 alkynylcarbonyl). Even more preferably, one of R_1 and R_2 is C_1-C_6 alkylcarbonyl and most preferably methylcarbonyl (acetyl).

Preferably, R_3 is alkyloxy (more preferably C_1-C_6 alkyloxy), alkenyloxy (more preferably C_2-C_6 alkenyloxy), alkynyloxy (more preferably C_2-C_6 alkynyloxy) or aryloxy (more preferably phenyloxycarbonyl). Even more preferably, R_3 is benzyloxy.

Particularly preferred transforming agents are where R_1 is H, R_2 is acetyl and R_3 is carboxyl (N-acetyl glycine) or benzyloxy (N-acetyl glycine benzyl ester) and where R_1 and R_2 are H and R_3 is benzyloxy (glycine benzyl ester).

For the avoidance of doubt, the transforming agents useful in the method of the present invention include physiologically acceptable salts and other derivatives of the above-mentioned compounds of Formula I which are converted to a compound of formula I under physiological conditions.

It will be understood that said transforming agents generally do not in themselves have anti-microbial properties, but merely potentiate the activity of anti-microbial agents.

Preferably, said bacterial strain has resistance to said anti-microbial agent.

The present invention also resides in the use of an agent having formula (I) in the manufacture of a medicament for increasing the sensitivity of a bacterial strain infecting or carried by a patient to an anti-microbial agent.

The invention further resides in a method of treating a patient infected by carrying a bacterial strain, comprising administering to said patient an amount of a transforming agent of formula (I) sufficient to render said strain more sensitive to an anti-microbial agent, and administering to said patient a therapeutically effective amount of said anti-microbial agent.

It will be understood that said patient may be a non-symptomatic carrier of the bacterial strain or said patient may be inflicted with a symptomatic clinical infection.

Administration of said transforming agent may be prior to, subsequent to or concomitant with the administration of the anti-microbial agent. However, said transforming agent is preferably administered together with or prior to said anti-microbial agent. In the case of concomitant administration, the transforming agent and anti-microbial agent may be administered in combination as a single medicament or as separate medicaments. Preferably, the transforming agent and the anti-microbial agent are administered in combination as a single medicament.

Administration may be by any known route eg. by intravenous, intramuscular, or intrathecal (spinal) injection, intranasal, topical administration as an ointment, salve, cream or tincture, oral administration as a tablet, capsule, suspension or liquid and nasal administration as a spray (eg. aerosol).

In each case said agent or combination of agentsmay be in admixture with one or more excipients, carriers, emulsifiers, solvents, buffers, pH regulators, flavourings, colourings, preservatives, or other commonly used additives in the field of pharmaceuticals as appropriate for the mode of administration.

Preferably, said agent is capable of increasing the sensitivity to the antimicrobial agent of at least one bacterial strain selected from methicillin resistant *Staphylococcus aureus*, coagulase-negative staphylococci and enterococci, vancomycin-resistant enterococci, *Clostridium difficile*, and *Streptococcus pneumoniae*. More preferably said agent is capable of increasing the sensitivity to the antimicrobial agent of at least one of methicillin resistant *Staphylococcus aureus* and vancomycin-resistant enterococci.

In particular, said agent is preferably capable of increasing the sensitivity of EMSRA-15, -16 and/or –17 to β-lactam (and analogous) antibiotics, and/or increasing the sensitivity of EMSRA-17 to glycopetptide antibiotics.

In each case, sensitivity is preferably increased to the level of a comparable non-resistant bacterial strain at a concentration of agent of 0.02M or less, more preferably 0.002M or less and most preferably 0.001M or less as determined by a standard antibiotic sensitivity test.

Preferably, the anti-microbial agent to which sensitivity is increased is selected from the group consisting of β-lactam (and analogous) antibiotics (eg. methicillin, piperacillin, flucloxacillin, cloxacillin, oxacillin, Augmentin, ofloxacillin, imipenam and merpenam), cephalosporins (eg. ceftazidime and cefuroxime) and glycopeptides (eg. vancomycin, teicoplanin, gentamicin and LY333328 (Ely Lily)).

It will be understood that two or more antimicrobial agents (from the same or preferably different classes) may be employed.

Methicillin-resistance in staphylococci

The staphylococcal cell wall plays an important role in the pathogenesis and treatment of infection. In Gram-positive bacteria, the cell wall consists of layers of peptidoglycan that are cross-linked by peptide bridges. Gram-negative bacteria have a thin peptidoglycan layer encapsulated by an outer cell membrane. Because of the uniqueness of the peptidoglycan structure and assembly, it is one of the preferred targets of antimicrobial agents, including antibiotics produced naturally by several types of microorganisms. The peptidoglycan of *Staphylococcus aureus* consists of linear sugar chains of

alternating units of N-acetylglucosamine and N-acetylmuramic acid substituted with a pentapeptide L-Ala – D-Glu – L-Lys – D-Ala – D-Ala. A characteristic of the cell wall of *S. aureus* is a pentaglycine cross-link that connects L-Lys to the D-Ala on the pentapeptide of a neighbouring unit, the terminal D-Ala being split off by transpeptidation. This flexible pentaglycine bridge allows up to 90% of the peptidoglycan units to be cross-linked, thus facilitating substantial cell-wall stability. In addition, the pentaglycine link acts as a recipient for staphylococcal surface proteins that are covalently anchored to it by a transpeptidase-like reaction. Surface proteins play an important role in adhesion and pathogenicity by interacting with host matrix proteins.

The major theory involving the mechanism of action of β-lactams concerns their structural similarity to the D-Ala – D-Ala carboxy-terminal region of the peptidoglycan pentapeptide. Penicillins, cephalosporins and other β-lactams, acylate the active site serine of cell wall transpeptidases, forming stable acylenzymes that lack catalytic activity. Inhibition of peptidoglycan synthesis by covalent binding of β-lactams to cell wall synthetic enzymes known as penicillin binding proteins (PBPs), allows autolysis in *S.aureus* mediated by endogenous autolytic enzymes. Although autolysis is less possible in MRSA, the *llm* gene encodes a lipophillic protein of 351 amino acid residues that is associated with decreased methicillin resistance accompanied by increased autolysis. Methicillin-sensitive *S. aureus* produce four major PBPs with molecular masses of about 85, 81, 75 and 45 kDa, respectively referred to as PBPs 1, 2, 3 and 4 (by convention, PBPs are numbered in order of diminishing molecular mass). Resistance to penicillin in *S. aureus* was originally acquired in the form of β-lactamases or penicillinases, now produced by about 90% of clinical isolates. The structural gene for β-lactamase, *blaZ*, and two regulatory genes, *blaI* and *blaRI*, usually reside on a transmissible plasmid, although chromosomal location has been identified in some strains. The induction of β-

lactamase is believed to be initiated by the binding of β-lactams to the transmembrane domain of a signal-transducing PBP encoded by *blaRI* (PBP3), leading ultimately to repressor degradation with loss of its DNA-binding properties, such that the transcription of *blaZ* is permitted. The means by which the BlaRI-penicillin complex causes repressor degradation is unclear, although it is thought that this could either result from, 1) conformational changes to BlaRI brought about by activation of a protease in the cytoplasmic domain by β-lactam binding, or 2) a repressor-inactivating protease encoded by a putative gene *blaR2* which the BlaRI-penicillin complex either activates or causes to be induced. β-lactamases catalyse the inactivation of penicillin and other β-lactams (depending on the class of β-lactamase) by covalently binding to the β-lactam ring. This is essentially the same reaction that occurs when β-lactams bind to the active site of PBPs except that the reaction is non-hydrolytic and not reversible. Some PBPs have detectable β-lactamase activity, including PBP 4 of *S. aureus*. However, high molecular weight PBPs (eg. PBPs 1, 2 and 3 in *S. aureus*) are mainly involved with peptidoglycan transpeptidation, whilst low molecular weight ones exhibit carboxypeptidase activity.

Methicillin-resistance in *S. aureus* and coagulase-negative staphylococci is defined by the production of a specific PBP, PBP2a, that has a reduced affinity for β-lactam compounds. The low affinity PBP2a, confers intrinsic resistance to virtually all β-lactam antimicrobial agents, including cephalosporins. PBP2a functions as a transpeptidase in cell wall synthesis in MRSA when high concentrations of β-lactams are present, which inhibits the activity of the normal PBPs, 1-4. PBP2a is encoded by the structural gene *mecA* located on the methicillin-resistant staphylococcal chromosome. Expression of PBP2a is controlled by two regulator genes on *mec* DNA, *mecI* and *mecRI*, located

upstream of *mecA*, which encode a *mecA* repressor protein and signal transducer protein, respectively. MRSA carrying intact *mecI* and *mecRI* together with *mecA*, are referred to as 'pre-MRSA'. Since intact *mecI* product strongly represses the expression of PBP2a, the pre-MRSA is apparently susceptible to methicillin. It has been hypothesised that removal of the repressor function for *mecA* is a prerequisite for constitutive expression of methicillin-resistance in *S. aureus* with *mec* DNA. There is homology between *mecI* and *blaI*, *mecRI* and *blaRI*, and the promoter and N-terminal portions of *blaZ* and *mecA*. This homology is strong enough that *blaI* can restore the normal inducible phenotype to isolates of *S. aureus*, which results in large amounts of constitutive PBP2a production because of the absence of or a defect in, the *mecI* locus. Increased PBP2a production may be associated with vancomycin-resistance (see below).

Subsequent to the discovery of PBP2a, it was realised that the phenotypic expression of methicillin-resistance did not correlate with the amount of PBP2a expressed. In 1983, it was shown that several additional genes independent of *mecA* are needed to sustain the high level of methicillin-resistance in MRSA. These genes were called *fem*, as they were thought to provide factors essential for methicillin-resistance, or *aux*, for auxiliary factors. While it was originally thought that the *fem* or *aux* factors represented additional genes recruited by staphylococci after the acquisition of *mecA* to further improve and consolidate methicillin-resistance and its homogeneity, it became increasingly clear that the *fem* genes were natural constituents of all staphylococci, and were involved in the formation of the pentapeptide bridge and modification of this bridge or the muropeptide. Synthesis of the pentaglycine bridge occurs at the membrane-bound lipid II precursor NAG-(β-1,4)-NAM-(L-Ala – D-Glu – L-Lys – D-Ala – D-Ala)-pyrophosphoryl-undecaprenol by sequential addition of glycine to the

ε-amino group of lysine, using glycyl-tRNA as donor, in a ribosome-independent fashion. Six *fem* genes (*femA*, *femB*, *femC*, *femD*, *femE*, *femF*) have been described. *femA* and *femB* are two closely related but distinct genes that form part of an operon. Both *femA* and *femB* have been shown to be involved with the formation of the pentaglycine bridge. FemA, the product of *femA* is responsible for adding glycines 2 and 3 to the bridge, whilst FemB, the product of *femB*, adds glycines 4 and 5. A hypothetical *femX* was proposed as being responsible for a protein that added the first glycine.

Other FemA,B-like factors were identified in staphylococci, such as Lif in *Staphylococcus simulans* and Epr in *Staphylococcus capitis*, which protect these organisms from their own glycyl-glycine endopeptidase. Three new genes, *fmhA, B* and *C,* were subsequently identified. These fem-like genes are responsible for introducing 1-2 serine residues into the pentapeptide bridge in coagulase-negative staphylococci and may, under certain conditions, incorporate serine residues into positions 3 or 5 in the bridge in some strains of *S. aureus*. *fmhB* was subsequently shown to be the postulated *femX*, which added glycine residues to position 1 in the pentaglycine interpeptide bridge.

Inhibition of the formation of the pentaglycine bridge reduces resistance to methicillin without affecting synthesis of PBP2′, resulting in β-lactam hypersusceptibility. Thus the pentaglycine bridge has an important function in maintaining cell wall stability, including resistance to antimicrobial agents.

Vancomycin resistance

Glycopeptide antibiotics are inhibitors of peptidoglycan synthesis. Unlike β-lactams and related antimicrobials, glycopeptides do not bind directly to cell wall biosynthetic enzymes (PBPs) but complex with the carboxy moiety of the

terminal D-alanine of the cell wall precursor pentapeptide. This blocks progression to the subsequent transglycosylation steps in peptidoglycan synthesis and interferes with the reactions catalysed by D,D-transpeptidases and D,D-carboxypeptidases necessary for the anchoring of the peptidoglycan complex.

With the first appearance of VRE, it was apparent that strains could be divided by their type and level of glycopeptide resistance. There are now seven genotypic classes to characterise glycopeptide-resistant enterococci: *van*A, found predominantly in *E. faecium* and *E, faecalis* that confers resistance to ≥ 256 mg/l of vancomycin and ≥ 32 mg/l of teicoplanin; *van*B, found in *E. faecium*, *E, faecalis* and *Streptococcous bovis* that confers resistance to between 4 and 1000 mg/l of vancomycin and ≤ 1.0 of teicoplanin; *van*C1 (*E. gallinarium*), *van*C2 (*E. casseliflavus*), *van*C3 (*E. flavescens*) that confers resistance to between 2 and 32 mg/l of vancomycin and ≤ 1.0 of teicoplanin; *van*D, which confers resistance to between 64 and 256 mg/l of vancomycin and 4 to 32 mg/l of teicoplanin in *E. faecium*; and *van*E, which confers resistance to 16 mg/l of vancomycin and 0.5 mg/l of teicoplanin in *E. faecalis*. VRE of VanA type provide the main model for achieving high-level vancomycin-resistance: instead of producing cell wall unit pentapeptides with D-Ala – D-Ala tails to which vancomycin and other glycopeptides bind, the *vanA* gene cluster is induced by glycopeptides to produce D-Ala – D-Lac tails to which vancomycin and teicoplanin do not bind. The *vanA* gene cluster is contained on a transposable element TN1546 and the *vanA* gene itself produces a 39 Kda protein located in the cytoplasmic membrane. This protein is a ligase that preferentially synthesises D-Ala – D-Lac. In addition to *vanA*, there are two other genes – *vanH*, which is a dehydrogenase enzymes that produces D-lac from pyruvate, and *van*X, which encodes a metallo-dipeptidase that

preferentially hydrolyses D-Ala - D-Ala. The transcriptional activation of *vanHAX* is regulated by the VanRS two-component regulatory system comprising of the genes *vanS*, the signal sensor, and *vanR*, the response regulator. The remainder of the *vanA* gene cluster includes two additional genes, *vanY* (a D,D-carboxypeptidase that cleaves terminal D-Ala from pentapeptide residues and can increase the level of glycopeptide resistance further by eliminating binding targets, ie. D-Ala – DS-Ala) and *vanZ* (which mediates increased resistance to teicoplanin).

The ultimate emergence of vancomycin-resistant MRSA has been anticipated since it was shown experimentally that *vanA* genes from VRE may be transferred into a recombinant-deficient *S. aureus*. However, this has not happened in practice with either *S.aureus* or coagulase-negative staphylococci. It appears that, in MRSA, vancomycin-tolerance does not occur without tolerance to ß-lactams and that tolerant strains of *S.aureus* causing endocarditis, are associated with increased mortality. Vancomycin-tolerance has also emerged in *Streptococcus pneumoniae* and tolerant strains are more easily transformed to high-level resistance. This appears to be mediated by DNA changes in a two-component sensor-regulator system (VncS-VncR) which mediates changes in gene expression related to cell-wall formation. Amino-acid sequences of VncS and VncR show 38% homology to the $VanS_B$-$VanR_B$ regulatory system associated with glycopeptide-resistance in vancomycin-resistant *E. faecalis* (VREF) and are probably relevant to MRSA. Indeed, overproduction of a 37kd cytoplasmic protein thought to be a D-lactate dehydrogenase analogous to VanH in VREF, has been associated with vancomycin-resistance in a strain of *S. aureus*. This staphylococcal D- lactate dehydrogenase may also be under signal-transduction control mechanisms similar to the two-component homologous regions in S. *pneumoniae* and

MRSA probably have sequences homologous to $VanS_B$-$VanR_B$/VncR-VncS. Vancomycin-resistance in MRSA has been achieved by other means rather than by the acquisition of new genetic elements, namely by altering cell wall composition, which is largely regulated by enzymes classically sensitive to penicillin (PBPs). Overproduction of PBP2a, a thickened cell wall containing a high glutaminenon-amidated component, and an increase in cell wall synthesis have all been cited as mechanisms. The appearance of a cell membrane dehydrogenase homologous to VanH in enterococci, has not yet been shown to be of importance in clinical strains, although there is a definite potential for high level vancomycin resistance to develop using this protein. Currently, the type of vancomycin-resistance in *S. aureus* is described as Intermediate or reduced which is difficult to detect by routine diagnostic methods. The main method of detection is by treatment failure.

Therapeutic use of teicoplanin is slightly controversial as it has not been approved for use in the USA and may select for vancomycin-resistant *S. aureus*. MRSA with reduced sensitivity to glycopeptides isolated from diabetic foot ulcers has been associated with use of teicoplanin and treatment failure has been associated with increased MICs of teicoplanin.

High concentrations of exogenous glycine are known to affect cell wall synthesis. Of more specific interest is the finding that glycine reduces the MIC of methicillin against MRSA: De Jonge and colleagues (Antimicrobial Agents and Chemotherapy (1996), **40**, pp1498-1503) used increasing concentrations of glycine in the growth medium, which resulted in peptidoglycan in which muropeptides with a D-Ala – D-Ala – terminus were replaced with D-Ala – glycine – terminating muropeptides. The authors concluded that the disappearance of D-Ala – D-Ala – terminating muropeptides in peptidoglycan

and the concomitant decrease in resistance, indicated a central role for D-Ala – D-Ala – terminating precursors in methicillin resistance.

Initial experiments with MRSA prevalent in the UK during the 1980s found that 2% glycine transformed all MRSA into methicillin-sensitive strains. This occurred only in the presence of glycine; cells were not permanently affected. In the presence of glycine, MRSA were also sensitive to cephalosporins and other β-lactam agents that were not hydrolysed by staphylococcal β-lactamase, i.e. penicillin-resistance was stable due to the production of this enzyme.

As far as the inventors are aware, the use of glycine as a transforming agent for the clinical treatment of MRSA has not been advanced. Nor has the use of glycine been investigated for the transformation of strains resistant to non-β-lactam antimicrobials, for example glycopeptide antimicrobials.

Examples

To find substances related to glycine that might have increased potency, various substances have been screened. Screening was carried out on Isosensitest agar (Oxoid, UK) in the manner of a standard antibiotic sensitivity test using 10 μg methicillin discs. The test organism was inoculated onto the agar surface at a concentration suitable to achieve confluent growth after 18 hours incubation at 30°C. A series of agar plates containing different concentrations of the test substance were used for each test organism. After incubation, zone diameters were compared with that achieved by the control plate for each test organism.

Glycine **(Comparative Example)**

Glycine anhydride **(Example 1)**

Di-glycine (glycylglycine) **(Example 2)**
Triglycine **(Example 3)**
N-acetylglycine (NAGly) **(Example 4)**
N-tris(hydroxymethyl)methyl glycine **(Example 5)**
N, N-di-methyl glycine **(Example 6)**
N, N-bis(2-hydroxymethyl) glycine **(Example 7)**
Ethyl glycine **(Example 8)**
D-2-(t-butyl) glycine **(Example 9)**
Glycine benzyl ester (GBE) **(Example 10)**
Glycinamide **(Example 11)**
N-carbamoylglycine (Hydantoic acid) **(Example 12)**
N-CBZ-glycine **(Example 13)**

All the above substances, including glycine itself transformed a reference MRSA and various selected MRSA (OMRSA), including the main epidemic type throughout the 1980s and early 90s, EMRSA-1.

Hydantoic acid had low-level active against vancomycin-resistant enterococci (VRE)

Table 1 shows the improved effect on methicillin sensitivity of glycine benzyl ester (GBE) (Example 10) on various patient isolated MRSA (L-series) and reference strains. At the time of isolation, the patient isolates were resistant to all clinically available β-lactams, cephalosporins, macrolides and gentamicin. There was variable sensitivity to tetracycline, trimethoprim, chloramphenicol, fusidic acid and rifampicin.

As can be seen from Table 1, glycine benzyl ester increased sensitivity to methicillin to a much greater extent than glycine. Even at 0.001M, an improved effect was observed over glycine at 0.2M (test 3 cf. test 1) for all strains.

Table 1

Isolate tested	MIC of methicillin (mg/l)			
		Glycine		GBE
	0.0 (Control)	0.02M (0.15%) (Test 1)	0.2M (1.5%) (Test 2)	0.001M (0.2%) (Test 3)
NCTC 12493	>256	0.12	0.06	0.015
L265	>256	64	8	2
L266	>256	64	8	2
L267	>256	32	8	2
L268	>256	32	8	2
L269	>256	16	4	1
L270	>256	8	4	1
L271	>256	8	4	2
L272	>256	16	2	1
L273	>256	8	4	1
L274	>256	16	4	2
L275	>256	32	8	2
L276	>256	16	4	2
L277	>256	8	4	2
L278	>256	64	16	4
L279	>256	64	2	2
L280	>256	16	4	2
L281	>256	32	4	2
L282	>256	32	4	2
L283	>256	32	4	2
L284	>256	32	2	2
L285	>256	32	4	2
L286	>256	32	4	2
L287	>256	32	4	2
L288	>256	32	4	2
L289	>256	32	4	2
L290	>256	32	4	2
L291	>256	32	4	2

L292	>256	32	4	2
L293	>256	32	4	2
L294	>256	32	4	2
MC01*	>256	32	4	2
JF1-32*	>256	32	4	2
DS09*	>256	32	4	2
SW2-32*	>256	32	4	2
PS3-32*	>256	32	4	2
ST11*	>256	32	4	2
SN31*	>256	32	4	2
CD40*	>256	32	4	2
E16-96**	>256	32	4	2
E15-97***	>256	32	4	2

*EMRSA-1; **EMRSA-16; ***EMRSA-15

In table 1, the target MIC for transformation is provided by the vancomycin-sensitive reference strain NCTC 12493, which has an MIC of vancomycin of 2 mg/l. 0.2 M glycine achieves this target in 50% of strains tested, compared to 0.02 M of glycylbenzyl ester which achieves complete transformation in 100% of strains tested.

Importantly, the usefulness of the agents of the present invention is not limited to increasing bacterial sensitivity to methicillin. The transforming effect of glycyl benzyl ester on two cephalosporins is shown in Table 2.

Table 2

Isolate of MRSA tested	MIC of ceftazidime or cefuroxime (mg/l) when grown with or without glycine benzyl ester (GBE):			
	Control		GBE (0.2%)	
	Ceftazidime	Cefuroxime	Ceftazidime	Cefuroxime
NCTC 12493	>256	>256	2	4
MC01*	>256	>256	2	4
JF1-32*	>256	>256	2	2
DS09*	>256	>256	2	2
SW2-32*	>256	>256	4	4
PS3-32*	>256	>256	4	4
ST11*	>256	>256	2	2
SN31*	>256	>256	4	4
CD40*	>256	>256	4	2
E16-96**	>256	>256	2	4
E15-97***	>256	>256	4	4

*EMRSA-1; **EMRSA-16; ***EMRSA-15

Glycyl benzyl ester transforms the MRSA tested to ceftazidime and cefuroxime sensitivity, thus making these two drugs that have never had useful activity against MRSA newly active against MRSA.

The potential for useful activity *in vivo*, is demonstrated in Table 3, which shows the MICs of methicillin in 1% human plasma for 19 patient-isolates of MRSA for glycine benzyl ester and glycine as a reference. Stored frozen plasma was pooled from five subjects.

Table 3

Isolate of MRSA tested	MIC of methicillin (mg/l) when grown in Moles (%) of glycine or GBE with or without 1% human plasma			
	Glycine (0.02M [0.15%])		GBE (0.00075M [0.15%])	
	No plasma (Control 1)	+ plasma (Test 1)	No plasma (Control 2)	+ plasma (Test 2)
L277	8	32	4	8
L278	64	256	8	16
L279	64	256	4	16
L280	16	64	4	8
L281	8	32	4	8
L282	32	256	4	16
L283	32	128	4	16
L284	32	256	2	8
L285	32	256	4	16
L286	32	64	4	8
L287	32	128	4	16
L288	32	128	2	16
L289	32	256	4	16
L290	32	128	4	8
L291	32	64	4	16
L292	32	256	4	32
L293	32	128	2	8
L294	16	128	2	8
5518*	8	32	2	4

*EMRSA-1

Human plasma may bind or otherwise inactivate foreign substances and good activity in plasma is indicative of good *in vivo* activity. Approximations from the above data suggest glycine is reduced in activity by about 75% and glycine benzyl ester by about 75% to 50%. This may be due to protein binding rather than enzymatic degradation, indicating the useful stability of the compound *in*

vivo. Again the increase in sensitivity to methicillin is significantly increased for glycine benzyl ester relative to glycine.

Table 4 shows the ability of glycine benzyl ester and N-acetyl glycine (NAGly) (Example 4) to transform MRSA with intermediate resistance to glycopeptides into glycopeptide-sensitive strains.

Table 4

MRSA tested	MIC of vancomycin or teicoplanin (mg/l) when grown in Moles (%) of GBE or NAGly of: Control	NAGly 0.001M	GBE 0.001M
MICs of vancomycin			
EMRSA-17 (VISA)			
L266	8	4	2
L266	8	2	1
NCTC 12493	0.5	0.25	0.12
MICs of teicoplanin			
EMRSA-16 (TISA)			
L265	32	4	1
L266	8	4	2
NCTC 12493	0.25	0.15	0.06

This data shows that glycine benzyl ester and N-acetyl glycine can restore the activity of vancomycin in vancomycin-intermediate-resistant MRSA (VISA) and teicoplanin in teicoplanin-intermediate-resistant MRSA (TISA), by reducing MICs to below the recognised resistant threshold of an MIC of 8 mg/l which defines intermediate resistance, at very low concentrations (0.001M).

The agents of the present invention are not limited to the reversal of resistance in *Staphylococcus*. The test strains in Table 5 are patient-isolates of vancomycin- and gentamicin-resistant *Enterococcus faecium*. At the time of isolation, they were commonly resistant to all clinically useable antimicrobial agents.

Table 5

Strain tested	MIC of vancomycin (mg/l) when grown in Moles (%) of glycine or glycine benzyl ester (GBE) of:			
		Glycine		GBE
	0.0 (Control)	0.02M (Test 1)	0.2M (Test 2)	0.02M (Test 3)
ATCC 29212	2	4	2	1
S317	128	32	4	2
S227	128	32	4	2
E267	128	16	4	2
E254	128	16	4	2
E297	128	8	2	2
S226	128	8	4	2
S283	64	8	2	2
S315	64	4	1	1
S497	64	8	2	1
E285	64	16	2	2
S556	64	32	2	1
S319	64	16	4	2
S302	64	8	4	2
S393	64	8	2	2
E271	64	8	2	2
S333	64	8	2	2
GBC	64	8	4	2
WBC	64	8	4	2
BBC	32	16	4	2
S337	32	4	2	2

In table 5, the target MIC for transformation is provided by the vancomycin-sensitive reference strain ATCC 29212, which has an MIC of vancomycin of 2 mg/l. 0.2 M glycine achieves this target in 50% of strains tested, compared to 0.02 M of GBE which achieves complete transformation in 100% of strains tested.

As previously mentioned, a common cause of auto-infection is due to *S. aureus* carried on the anterior nares. The data in Table 6 show that glycyl benzyl ester increases the sensitivity of already sensitive bacteria to methicillin (and by implication other related antibiotics such as flucloxacillin). The transforming agents of the present invention may also be used in combination with a suitable antimicrobial to eliminate nasal carriage of MSSA prior to cardiac surgery or other invasive procedures carrying a high risk of auto-infection.

Table 6

Isolate tested	MIC of methicillin (mg/l) when grown in Moles (%) of glycine or GBE with or without 1% human plasma		
		GBE (0.00075M [0.15%])	
	No plasma (Control 1)	No plasma (Test 1 and control 2)	plasma (1%) (Test 2)
LHS77	<0.25	<0.25	<0.25
LHS78	0.25	<0.25	1
LHS79	0.5	<0.25	0.25
LHS80	0.25	<0.25	0.25
LHS81	<0.25	<0.25	<0.25
LHS82	0.5	<0.25	4
LHS83	0.25	<0.25	0.25
LHS84	0.25	<0.25	2
LHS85	0.25	<0.25	0.5
LHS86	0.25	<0.25	0.25
LHS87	0.5	<0.25	4
LHS88	0.25	<0.25	0.5
LHS89	0.25	<0.25	0.5
LHS90	<0.25	<0.25	<0.25
LHS91	<0.25	<0.25	<0.25
LHS92	0.5	<0.25	1
LHS93	0.25	<0.25	0.5
LHS94	0.25	<0.25	0.5
5518*	>256	<0.25	0.5

*EMRSA-1

For clinical use, the agents may be administered systemically (eg. intravenously) for serious systemic infections such as septicaemia. However, it is anticipated that one of the principle uses of the agents will be topical administration for the subsequent treatment of local infections, or as part of a program to eliminate resistant bacteria from a carrier prior to surgery, for example, to prevent dissemination of infection before it arises.

The following is a non-exhaustive list of antibiotics which may be incorporated with the transforming agents of the present invention and their preferred routes of administration:-

Oral administration: amoxycillin, ampicillin, Augmentin

IV administration: vancomycin, meropenam, flucloxacillin, cloxacillin, ofloxacillin, cefuroxime, ceflazidime,

IM administration: teicoplanin

Topical: flucloxacillin

General formulation considerations

As far as systematic administration is concerned, co-formulation is generally preferred if the half-lives of the transforming agent and the antimicrobial are comparable. For example the penicillins generally have a half life of about 1.5 to 2 hrs and are administered 3 to 4 times daily. On the other hand teicoplanin has a half life of 12 hrs and is usually administered once a day. Thus, the transforming agent should be selected to have a corresponding half life, or alternatively be administered separately on a different dosing regimen.

In general, the transforming agent should be in sufficient concentration to achieve *in vivo* levels that will effect transformation in the target bacteria during approximately the same period as the half life of the antimicrobial. Of course it will be understood that the actual concentration of the transforming agent is not relevant to the concentration of the antimicrobial in the formulation.

Medicament Example 1

Glycine benzyl ester and flucloxacillin is mixed with paraffin wax, softisan [TM], hydroxypropyl methyl cellulose, polyglyceryl-4-caprate and glycerine to give an ointment containing 0.2wt% of the ester and 1 wt% of flucloxacillin.

Treatment regime

The ointment is rubbed into the infected area 3 to 4 times daily until the infection is eliminated.

Medicament Example 2

N-acetyl glycine and cefuroxime are mixed with an inert carrier liquid to give a 1% w/v of each active and dosed to a spray applicator.

Treatment regime

The medicament is sprayed intranasally 3 to 4 times daily for five day prior to surgery (or during an hospital outbreak) to eliminate anterior nares carriage of *S. aureus*. Treatment can be continued after surgery if desired or if there is re-inoculation of the carriage site.